IMMUNOSTIMULANT BACTERIAL MEMBRANE FRACTIONS IN THE TREATMENT OF CANCERS

The present invention relates to the use of a membrane fraction of Gram-negative bacteria, in particular of Klebsiella *pneumoniae*, for preparing a pharmaceutical composition which is immunostimulant and/or capable of inducing an antitumor immune response and which is intended, in particular, for treating and preventing cancers. The invention also comprises methods for preparing said membrane fractions and also pharmaceutical compositions containing them, in particular combined with anticancer compounds.

The transformation of a normal cell into a malignant cell is the result of many different events which may occur spontaneously, such as mutations or gene rearrangements, or be induced by chemical, physical or viral agents.

Tumors are infiltrated by immunocompetent cells, in particular lymphocytes, dendritic cells and macrophages.

Tumor-associated macrophages (TAMs) originate from the blood circulation and are recruited to the tumor site by cytokines. TAMs bind to the tumor cells via glycoproteins, sugars and phospholipids and proliferate at the tumor site (J. Natl. Cancer Inst., 1998, 90:1583). There, they secrete many cytokines which contribute to their antitumor activity. Among the most important are TNF-α and IL-12.

The antitumor activity of TNF-α has been demonstrated in experimental models in mice (Beyaert R. and Fiers W., Cytokines, chapter 24, 335-360 Academic Press. 1998) and has been tested in humans for treating bladder cancers: alone, it has moderate activity

(Steinberg et al., Ann. Oncol., 1992, 3,741-745; Eur. Urol. 1992, 22:112).

The production of IL-12 by activated macrophages serves to modulate the immune response by promoting the formation of Th1-type CD4+ T lymphocytes which produce IL-2 and IFN-γ. The inhibitory activity of IL-12 on angiogenesis and tumor regression is well known and appears to be linked to the induction of IFN-γ, which stimulates the production of IP-10 (interferon-inducible protein-10) and of MIG (monokine induced by IFN-γ) (J. Natl. Cancer Inst., 1998, 90:1583).

BCG (Bacille Calmette Guérin) therapy is used to prevent the recurrence of certain types of bladder cancer. The mechanism of action currently proposed is based on the production of cytokines: early release of inflammatory cytokines (TNF-α, IL-1, IL-6, IL-8) and, secondly, production of IL-2 and of IFN-γ (Th1 response), then later of IL-4, of IL-5 and of IL-10 (Th2 response). Finally, there occurs a phase of cell activation with amplification of cytotoxic populations (Patard et al., Progrès en Urologie, 1998, 8,415-421).

However, BCG therapy does not only have advantages, since the effectiveness sometimes observed is at the expense of a morbidity which is also greater. In addition, there are contraindications for BCG therapy: active tuberculosis (but not prior tuberculosis), immunosuppression (HIV, transplantation, etc.), prior systemic reaction to BCG (hepatitis, pneumonia, BCGitis), steroid treatments. Furthermore, resistances or recurrences exist after BCG therapy.

The membrane fraction of K. pneumoniae I145 goes into the composition of a pharmaceutical preparation which prevents the occurrence and recurrence of respiratory infections of bacterial origin and which has been used in humans for 20 years. For this reason, there has been

enough time to assess the nontoxicity of the product. The set of data cited above shows that there exists, today, a need to have novel immunostimulants free of toxic activity. Such immunostimulants would be of great value for treating certain types of cancer.

Surprisingly, the authors of the present invention have demonstrated that membrane fractions of a Gram-negative bacterium, especially Klebsiella *pneumoniae* (named FMKp), in particular membrane fractions obtained using the methods as described hereinafter in the examples, have the desired immunostimulant properties.

The inventors have shown, surprisingly, that the FMKp or one of its major constituents, the OmpA outer membrane protein named P40 (as described in patent applications WO 95/27787 and WO 96/14415) is capable not only of stimulating the proliferation of human blood mononucleated cells, thus demonstrating its immunostimulant activity, but also of inducing, in particular by monocytes, the production of TNF-α and of IL-12, which are cytokines involved in the antitumor immune response.

Thus, the subject of the present invention is the use of a membrane fraction of Gram-negative bacteria, in particular of Klebsiella *pneumoniae*, as a compound which is immunostimulant and/or capable of inducing an antitumor immune response, or for preparing a pharmaceutical composition which is immunostimulant and/or capable of inducing an antitumor immune response, this being whatever the mode of administration in vivo chosen (enteral or parenteral route).

In the present invention, the term "immunostimulant compound" or "immunostimulant pharmaceutical composition" is intended to denote a compound, or a

pharmaceutical composition, capable of increasing a nonspecific immune response.

In the present invention, the expression "compound capable of inducing an antitumor immune response" or "pharmaceutical composition capable of inducing an antitumor immune response" is intended to denote a compound, or a pharmaceutical composition, capable, in particular, of increasing the effectiveness of an anticancer compound or increasing the effectiveness of an anticancer treatment, such as for example treatment by radiotherapy.

The invention also relates to the use as claimed in the invention, characterized in that the membrane fraction comprises at least membrane fractions of two different strains of bacteria.

In the present invention, the expression "membrane fraction of a bacterium" is intended to denote any purified or partially purified membrane fraction or extract which is obtained from a culture of said bacterium and for which the method of preparation comprises at least one step for lysing the bacteria obtained after culturing and one step for separating the fraction containing the membranes of said bacteria from the total lysate obtained after the lysis step, in particular by centrifugation or filtration.

In the present invention, the expression "membrane fraction of a bacterium when said bacterium is Klebsiella *pneumoniae*" is also intended to denote the P40 protein, which is the active fraction of the membrane fraction of Klebsiella *pneumoniae*, of amino acid sequence SEQ ID No. 2, or a fragment thereof.

According to the invention, the membrane fractions may be prepared according to the methods known to those skilled in the art, such as for example the method

described by Haeuw J.F. et al. (Eur. J. Biochem, 255, 446-454, 1998).

According to one particular embodiment, the invention relates to a use as claimed in the invention, characterized in that the membrane fraction is prepared using a method comprising the following steps:

a) culturing of said bacteria in a culture medium which allows their growth, followed by centrifugation of said culture;
b) where appropriate, deactivation of the lytic enzymes of the bacterial pellet obtained in step a), then centrifugation of the suspension obtained;
c) extraction and elimination of the non-membrane-bound proteins and of the nucleic acids of the pellet obtained in step a) or b) with at least one cycle of washing the pellet in an extraction solution;
d) digestion of the membrane pellet obtained in step c) in the presence of proteolytic enzymes, followed by centrifugation;
e) at least one cycle of washing the pellet obtained in step d) in a physiological solution and/or in distilled water; and
f) ultrasonication of the pellet obtained in step e).

Step b) for deactivating the lytic enzymes of the bacterial pellet obtained in step a) may be carried out using any known method for deactivating enzymes, such as, in particular, by heating the resuspended bacterial pellet to a temperature preferably close to 100°C, or by adding an inhibitor of the activity of these enzymes.

Step c) for extracting and eliminating the non-membrane-bound proteins and the nucleic acids of the pellet obtained in step a) or b) may be carried out, for example, with at least one cycle of washing the pellet in an extraction solution corresponding to the

addition of a hypertonic solution (extraction solution), preferably a saline solution with a molarity close to 1 M, followed, after a period of contact sufficient for the desired effect, by centrifugation of the suspension obtained and elimination of the supernatant obtained after said centrifugation, this washing cycle possibly being reproduced several times.

Step d) for digesting the membrane pellet obtained in step c) may be carried out in the presence of a solution of proteolytic enzymes, such as for example trypsin, chymotrypsin or any known enzyme with proteolytic activity, the conditions of the reaction, pH of the solution, and temperature and duration of the reaction preferably being adjusted to the optimal conditions for the activity of the enzyme(s) chosen, followed by a centrifugation, this digestion cycle possibly being reproduced several times with the same enzyme or the same combination of enzymes, or with a different enzyme for each digestion cycle carried out.

Step e) for washing the pellet obtained in step d) is carried out by taking the pellet up in a physiological solution or in distilled water, followed, after a sufficient period of contact, by a centrifugation, this washing cycle possibly being reproduced several times.

Finally, the objective of step f) for ultrasonicating the pellet is, in particular, to disintegrate and homogenize the membrane fraction obtained at the end of step e). The ultrasonication conditions (duration and power) will be determined by those skilled in the art depending, for example, on the amount of membrane fraction to be treated.

According to another particular embodiment, the invention relates to a use as claimed in the invention, characterized in that the membrane fraction is prepared using a method comprising the following steps:

a) culturing of said bacteria in a culture medium which allows their growth, followed, where appropriate, by centrifugation;

b) freezing of the culture medium or of the pellet obtained in step a), followed by thawing and drying of the cells;

c) elimination, using a DNase, of the nucleic acids from the dried cells obtained in step b), which have been resuspended;

d) grinding of the cells obtained in step c) and clarification of the suspension obtained;

e) precipitation, in acid medium, of the suspension obtained in step d) and elimination of the pellet;

f) neutralization of the supernatant obtained in step e) containing the membrane suspension, followed by dialysis and concentration of the membrane suspension; and

g) sterilization of the concentrated membrane suspension obtained in step f).

The conditions for freezing in step b) of the method below will, of course, be determined by those skilled in the art depending on the initial amount of pellet to be treated, preferably carried out at 4°C for at least 48 hours for the equivalent of 1 kg of dried cells.

In step c), the nucleic acids are eliminated, for example, by adding a DNase at a final concentration of 5 mg/ml of a suspension of cells at a concentration equivalent to 5% of dried cells.

The grinding of the cells obtained in step c) may be carried out using any system or apparatus known to those skilled in the art for grinding cells, such as presses or preferably such as Manton Gaulinet loop grinding for 30 minutes.

The clarification of the suspension obtained after grinding may be carried out using any system or apparatus known to those skilled in the art for clarifying ground bacterial cell material, such as the Sharpless system.

Step e) for precipitating, in acid medium, the suspension obtained in step d) may be carried out, for example, with acetic acid. The precipitation is followed by elimination of the pellet using, for example, a system of the Sharpless type and by recovery of the supernatant.

Step f) consists of a step in which the supernatant, obtained after precipitation in acid medium, is neutralized, diluted, dialyzed and then concentrated.

Finally, the last step consists of a step for sterilizing the membrane fraction concentrate obtained in the preceding step, for instance by heating at 121°C for approximately 35 minutes, for example.

The invention relates particularly to the use as claimed in the invention, characterized in that the membrane fraction is the Klebsiella *pneumoniae* P40 protein of sequence SEQ ID No. 2, a fragment thereof or a homologous protein, the sequence of which exhibits a percentage identity of at least 80%, preferably 90%, 95% and 99%, with the sequence SEQ ID No. 2, said fragments or said homologous protein being capable of inducing immunostimulant and/or antitumor activity.

For the purposes of the present invention, the term "percentage identity", "degree of identity" or "level of identity" between two nucleic acid or amino acid sequences is intended to denote a percentage of nucleotides or of amino acid residues which are identical between the two sequences to be compared, obtained after the best alignment, this percentage

being purely statistical and the differences between the two sequences being distributed randomly and over their entire length. Sequence comparisons between two nucleic acid or amino acid sequences are conventionally carried out by comparing these sequences after having optimally aligned them, said comparison being carried out by segment or by "window comparison" in order to identify and compare local regions of sequence similarity. The optimal alignment of the sequences for comparison may be produced, besides manually, by means of the local homology algorithm of Smith and Waterman (1981) [Ad. App. Math. 2:482], by means of local homology algorithm of Neddleman and Wunsch (1970) [J. Mol. Biol. 48:443], by means of the similarity search method of Pearson and Lipman (1988) [Proc. Natl. Acad. Sci. USA 85:2444], by means of computer software using these algorithms (GAP, BESTFIT, FASTA and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI, or with the BLAST N or BLAST P comparison software).

The percentage identity between two nucleic acid or amino acid sequences is determined by comparing these two sequences which have been optimally aligned by window of comparison in which the region of the nucleic acid or amino acid sequence to be compared may comprise additions or deletions with respect to the reference sequence for optimal alignment between these two sequences. The percentage identity is calculated by determining the number of identical positions for which the nucleotide or amino acid residue is identical between the two sequences, dividing this number of identical positions by the total number of positions in the window of comparison and multiplying the result obtained by 100, so as to obtain the percentage identity between these two sequences.

For example, use may be made of the BLAST program, "BLAST 2 sequences", which is available on the site

http://www.ncbi.nlm.nih.gov/gorf/bls.html, the parameters used being those given by default (in particular, for the "open gap penalty" parameter :5 and the "extension gap penalty" parameter :2; the matrix chosen being, for example, the "BLOSUM 62" matrix proposed by the program), the percentage identity between the two sequences to be compared being calculated directly by the program.

The expression "fragment of P40 protein" is intended to denote, in particular, any fragment of amino acid sequence included in the amino acid sequence of the P40 protein, which is capable of increasing a nonspecific immune response and/or capable of inducing an antitumor immune response, and which comprises at least 5 amino acids, preferably at least 10 amino acids, or more preferably at least 15 amino acids.

Of course, said P40 protein, or fragments thereof, may be obtained by chemical synthesis or in the form of recombinant peptides.

The methods for preparing recombinant peptides are, today, well known to those skilled in the art and will not be developed in the present description. Among the cells which may be used for producing these recombinant peptides, mention should, of course, be made of bacterial cells (Olins P.O. and Lee S.C., 1993, Recent advances in heterologous gene expression in E. coli. Curr. Op. Biotechnology 4:520-525), but also yeast cells (Buckholz R.G., 1993, Yeast Systems for the Expression of Heterologous Gene Products. Curr. Op. Biotechnology 4:538-542), as well as animal cells, in particular mammalian cell cultures (Edwards C.P. and Aruffo A., 1993, Current applications of COS cell based transient expression systems. Curr. Op. Biotechnology 4, 558-563), but also insect cells in which methods implementing, for example, baculoviruses may be used (Luckow V.A., 1993, Baculovirus systems for the

expression of human gene products. Curr. Op. Biotechnology 4, 564-572).

A subject of the invention is also the use as claimed in the invention, characterized in that the pharmaceutical composition also comprises an agent for vehiculing said membrane fraction in a form which makes it possible to improve its stability and/or its immunostimulant activity and/or its capacity to induce an antitumor immune response, such as in the form of an emulsion of the oil-in-water or water-in-oil type, or in the form of a particle of the liposome, microsphere or nanosphere type, or any type of structure which enables said membrane fraction to be encapsulated and presented in particulate form.

Also included in the present invention is the use as claimed in the invention, characterized in that the pharmaceutical composition also comprises an agent for potentiating the immunostimulant activity and/or the antitumor immune response of said membrane fractions.

Among said agents for potentiating the immunostimulant activity and/or the antitumor immune response of said membrane fractions, cytokines and cellular compounds are preferred.

Among cytokines, mention may be made, without being limited thereto, of: IL-2, IL-12, IL-18, IFN-γ and IFN-α.

Among cellular compounds, nucleic acids, compounds of the ribosome family or proteins of the heat-shock protein family are in particular preferred.

Also included in the present invention is the use as claimed in the invention, characterized in that the pharmaceutical composition also comprises a potentiating agent which makes it possible to regulate

the immunostimulant activity and/or the antitumor immune response of said membrane fractions.

Among said potentiating agents which make it possible to regulate the immunostimulant activity and/or the antitumor immune response of said membrane fractions, hormones and growth factors are preferred.

Among hormones, mention may be made, but without being limited thereto, of β-hCG.

Among growth factors, mention may be made, but without being limited thereto, of: EGF, IGF-1, IGF-2, GM-CSF and G-CSF.

The subject of the invention is also the use as claimed in the invention, for preparing a pharmaceutical composition intended to be administered in combination with an anticancer treatment, in particular an anticancer treatment by chemotherapy (mono- or polychemotherapy) and/or radiotherapy.

According to the invention, the preparation of the pharmaceutical composition is intended to be administered via the enteral or parenteral route, and simultaneously with, separately from or spread out over time with the anticancer treatment.

The invention also comprises the use as claimed in the invention, for preparing a pharmaceutical composition comprising a compound with anticancer activity combined with said membrane fraction.

Many compounds with anticancer activity may thus be combined with said membrane fraction which is immunostimulant and/or capable of inducing an antitumor immune response.

Among these compounds, mention may in particular be made, but without being limited thereto, of protease inhibitors or compounds with anti-angiogenic activity, such as for example:

- protease inhibitors such as TIMPs;

or the following compounds with anti-angiogenic activity: angiostatin, endostatin, MCP-1, IP-10 and PF-4, and also anti-VEGF, anti-angiogenin, anti-aFGF and anti-bFGF antibodies, antisense sequences or peptides.

Thus, the invention relates to the use as claimed in the invention, characterized in that said combined anticancer treatment is a chemotherapeutic treatment comprising a protease inhibitor or a compound with anti-angiogenic activity.

The subject of the invention is also the use as claimed in the invention, for preparing a pharmaceutical composition intended to prevent or treat cancers, in particular bladder cancers, prostate cancers, colon cancers, liver cancers or malignant melanomas.

In another aspect, the invention relates to a method for preparing a membrane fraction of Gram-negative bacteria, in particular Klebsiella *pneumoniae*, characterized in that it comprises the following steps:

a) culturing of said bacteria in a culture medium which allows their growth, followed by centrifugation of said culture;
b) where appropriate, deactivation of the lytic enzymes of the bacterial pellet obtained in step a), then centrifugation of the suspension obtained;
c) extraction and elimination of the non-membrane-bound proteins and of the nucleic acids of the pellet obtained in step a) or b) with at least one cycle of washing the pellet in an extraction solution;

d) digestion of the membrane pellet obtained in step c) in the presence of protease enzymes, followed by centrifugation;
e) at least one cycle of washing the pellet obtained in step d) in a physiological solution and/or in distilled water; and
f) ultrasonication of the pellet obtained in step e).

The invention also comprises the method for preparing a membrane fraction of Gram-negative bacteria, in particular Klebsiella *pneumoniae*, characterized in that it comprises the following steps:

a) culturing of said bacteria in a culture medium which allows their growth, followed, where appropriate, by centrifugation;
b) freezing of the culture medium or of the pellet obtained in step a), followed by thawing and drying of the cells;
c) elimination, using a DNase, of the nucleic acids from the dried cells obtained in step b), which have been resuspended;
d) grinding of the cells obtained in step c) and clarification of the suspension obtained;
e) precipitation, in acid medium, of the suspension obtained in step d) and elimination of the pellet;
f) neutralization of the supernatant obtained in step e) containing the membrane suspension, followed by dialysis and concentration of the membrane suspension; and
g) sterilization of the concentrated membrane suspension obtained in step f).

The membrane fractions which can be obtained using said methods of course form part of the invention.

The titer of proteoglycan of the membrane fractions which can be obtained using said methods, which proteoglycan is the active principle of the FMKp, which

titer is represented by the sum of the galactose and protein contents, is preferably:
- for the galactose : between 1.2 g/l and 3.4 g/l;
- for the proteins : between 7.5 g/l and 14.9 g/l.

More preferably, this titer will be:
- for the galactose : between 1.8 g/l and 2.6 g/l;
- for the proteins : between 9.3 g/l and 11.7 g/l.

The invention also relates to the pharmaceutical compositions comprising a membrane fraction which can be obtained using the methods as claimed in the invention.

Also included in the present invention are the pharmaceutical compositions comprising a membrane fraction of a Gram-negative bacterium, in particular of Klebsiella *pneumoniae*, characterized in that it is combined with an anticancer treatment by chemotherapy and/or by radiotherapy.

The term "membrane fraction" is herein intended to denote any membrane fraction of the Gram-negative bacterium as defined above, including that which can be obtained using the methods as claimed in the invention and the P40 protein or a fragment thereof.

Preferably, the invention relates to a pharmaceutical composition as claimed in the invention, characterized in that it contains an anticancer compound as a combination product for use which is simultaneous, separate or spread out over time, in particular an anticancer compound chosen from protease inhibitors or from compounds having anti-angiogenic activity.

Preferably, said pharmaceutical compositions as claimed in the invention may also comprise agents such as vehicles, agents capable of potentiating and/or of regulating the immunostimulant activity and/or the

antitumor immune response of said membrane fractions as defined above.

The legends to the figures and examples which follow are intended to illustrate the invention without in any way limiting the scope thereof.

Legends to the figures:

Figure 1 : Proliferation of PBMC in the presence of FMKp - Dose-response study

The mononucleated cells (PBMC) are obtained by separation with the aid of a solution of Ficoll-sodium metrizoate, using total blood. The PBMC are then seeded in a proportion of 10 000 cells/well in the presence of stimulating agents, in a total volume of 200 µl. After incubation for 72 h, the proliferation is objectified by adding tritiated thymidine. The results are expressed as stimulation index = [cpm PBMC + stimulus]/ [cpm PBMC without stimulus (= RPMI medium + 10% SVF)].

Figure 2 : Proliferation of PBMC in the presence of FMKp - Reproducibility of the effect on several donors (FMKp at 250 µg/ml).

Figure 3 : Production of TNF-α by blood monocytes

The monocytes are cultured in RPMI 1640 medium + 10% SVF and in the presence of various concentrations of product. The cells are incubated in an incubator at 37°C in an atmosphere containing 5% of CO_2. Culture conditions : 200 000 cells/well, incubation for 18 h. After incubation, the culture plates are centrifuged and the supernatants are aliquoted and stored at -80°C until they are assayed. The concentrations of cytokines present in the culture supernatants are determined by ELISA (Enzyme-Linked ImmunoSorbent Assay) : Predicta kit from Genzyme (detection threshold at 3 pg/ml).

Figure 4 : Production of IL-12 p70 (biologically active) by blood monocytes.

The monocytes are cultured in RPMI 1640 medium + 10% SVF and in the presence of various concentrations of product. The cells are incubated in an incubator at 37°C in an atmosphere containing 5% of CO_2. Culture conditions : 500 000 cells/well, incubation for 24 h. After incubation, the culture plates are centrifuged and the supernatants are aliquoted and stored at -80°C until they are assayed. The concentrations of cytokines present in the culture supernatants are determined by ELISA : Endogen antibody pair (detection threshold at 15 pg/ml).

Example 1 : Production of the membrane fraction of K.*pneumoniae* (FMKp)

Method No. 1

The extraction of the K. pneumoniae I145 membranes from the centrifugation pellet from the step is preferably preceded by a step for destroying the lytic enzymes of the cellular components contained in the pellet, for example by heating the pellet to 100°C, optionally after redissolving it.

The actual extraction of the membranes from the centrifugation pellet is preferably carried out by treating the cellular components of the pellet, after optional destruction of the lytic enzymes, with a saline solution, for example 1 M sodium chloride, one or more times, then centrifuging the suspension obtained, preferably at 20 000 g; the supernatant from this centrifugation, which is eliminated, contains the nonmembrane impurities such as proteins and nucleic acids, while the pellet contains the membranes.

After separation of the saline solution containing the impurities, the membranes are digested in the presence of proteolytic enzymes, preferably trypsin and chymotrypsin, in solution at pH 8, at 37°C for 4 hours.

After digestion, the solution is homogenized by ultrasonication. The product thus obtained constitutes the membrane fraction named FMKp.

The supernatant obtained is centrifuged again under the same conditions, preferably at 140 000 g.

Preparation of the membrane-bound glycopeptides

This fraction is prepared from the pellet obtained by centrifugation at 40 000 g for 20 minutes. Said pellet is resuspended in physiological saline and then this suspension is brought to 100°C for 10 minutes in a waterbath of boiling water so as to deactivate the lytic enzymes. After cooling, the suspension is centrifuged for 30 min at 20 000 g. The pellet obtained is extracted twice with 1M NaCl in order to eliminate the proteins and the nucleic acids. The membranes are recovered by centrifugation for 30 minutes at 20 000 g.

They are then subjected to digestion by trypsin at pH 8 and at 37°C for 4 hours, then by chymotrypsin under the same conditions.

The membranes are then recovered by centrifugation at 2 000 g for 30 minutes, washed with physiological saline and then distilled water and subjected to 15-minute disintegration by ultrasound.

Method No. 2

After thawing at +4°C for a minimum of 48 h, 1 kg of dry K. pneumoniae cells is resuspended at 5% dry cells. DNase is added at 5 mg/l. Next, Manton Gaulin loop grinding is carried out for 30 min, followed by a clarification of a Sharples at 50 l/h, and then

precipitation with acetic acid at pH = 4.2 + 0.1 for 30 min. The pellet is eliminated (Sharples at 25 l/h) and the supernatant is neutralized and diluted to twice the initial volume with osmosed water. Dialysis at constant volume is then performed on PUF 100 up to 800 Ωcm, followed by concentration of the membrane suspension (MS) thus obtained, to 11 l/kg of dry cells. The MS is then autoclaved at +121°C for 35 min and can be stored at +4°C for 6 weeks.

Characteristics of the FMKp

By definition, the titer of proteoglycan, which is the active principle of the FMKp, is equal to the sum of the galactose and protein contents.

- galactose : on average 2.2 g/l
- proteins : on average 10.5 g/l

Example 2 : Proliferation of PBMC from human blood

The results obtained show that, surprisingly, the FMKp triggers PBMC proliferation. This effect is dose-dependent and maximal for 2.5 mg/ml of FMKp (Figure 1). Moreover, this effect is reproducible (Figure 2).

Example 3 : Production of cytokines by monocytes purified from human blood

Human monocytes are obtained from the mononucleated cells (lymphocytes, monocytes, NK cells, etc.) isolated beforehand from total human blood. The production of monocytes is based on the expression, in large amount, of the CD14 surface antigen on the cells. The separation is a positive selection. The effectiveness of the magnetic separation of the monocytes is then evaluated by flow cytometry, labeling with a fluorescein isothiocyanate (FITC) - coupled CD13 antibody: the cell suspension then contains 94 to 97% of monocytes.

The results from in vitro studies demonstrate that, interestingly, the FMKp is an immunostimulant which

induces the proliferation of PBMC from human blood with a direct effect on the monocytes : production of TNF-α (Figure 3) and of IL-12 p70 (Figure 4). It is noteworthy that the recombinant P40 protein (rP40), the OmpA of K. pneumoniae, is also capable of stimulating the production of TNF-α (Figure 3) and of IL-12 p70 (Figure 4) by human monocytes.